International Journal of Infectious Diseases (2004) **8**, 339–345

http://intl.elsevierhealth.com/journals/ijid

Rapid diagnosis of rabies in humans and animals by a dot blot enzyme immunoassay

Shampur Narayan Madhusudana*, Joel Prem Vasanth Paul, Venugopal Karavattu Abhilash, Mooriyath Sukumaran Suja

Department of Neurovirology, National Institute of Mental Health and Neurosciences (NIMHANS), PB No. 2900, Hosur Road, Bangalore 560029, India

Received 28 October 2003; received in revised form 3 February 2004; accepted 4 February 2004
Corresponding Editor: Jane Zuckerman, London, UK

KEYWORDS
Rabies;
Rabies diagnosis;
Dot blot immunoassay

Summary

Objectives: The presently advocated tests for rapid diagnosis of rabies, such as the fluorescent antibody test (FAT) are expensive and require expertise to carry out and interpret the results. In this study, a simple direct dot blot enzyme immunoassay (DIA) has been developed and evaluated to detect the rabies antigen in brain specimens of animals and humans. The utility of this test in the ante-mortem diagnosis of human rabies has also been evaluated.
Methods: Brain homogenates of suspected rabid animals (n = 250), humans (n = 16) and clinical samples like saliva (n = 12) and cerebrospinal fluid (CSF) (n = 12) were directly spotted on polyvinylidene difluoride membrane (PVDF) and the absorbed rabies nucleoprotein antigen was detected using biotinylated antinucleoprotein antibody followed by treatment with streptavidin peroxidase conjugate and color development with diamino benzedine (DAB). Rabies-infected and normal mouse brain homogenates were used as positive and negative controls, respectively. The results of this test were evaluated with fluorescent antibody technique (for brain samples) and mouse inoculation test (for saliva and CSF samples).
Results: A distinct dark brown color was seen in the positive control and all positive samples, while there was no color development with either the negative control or the negative samples. The concordance between the fluorescent antibody test (FAT) and dot immunoassay was 98.4% for brain samples, 83.3% for saliva and 91.6% for CSF samples. The specificity of the test was found to be 100%.

* Corresponding author. Tel.: +91 80 699 5128/5129; fax: +91 80 656 4830.
E-mail address: mshampur@hotmail.com (S.N. Madhusudana).

doi:10.1016/j.ijid.2004.02.006

Conclusions: The dot blot enzyme immunoassay (DIA) test described here is a sensitive, specific and rapid test for the post-mortem diagnosis of rabies in animals and humans. The utility of this test for the ante-mortem diagnosis of rabies needs to be further evaluated.

Introduction

Human and animal rabies continues to be a significant public health problem in many Asian, South American and African countries. According to the latest report from the World Health Organization, about 50,000 human deaths are reported worldwide every year and more than 80% of these cases occur in Asian countries.[1] India alone accounts for 30,000 human deaths per year.

Although human rabies has been eliminated or controlled to a great extent in North America and Europe, rabies in wild animals and bats poses a significant threat to human populations in these regions. The Australian continent, which was hitherto considered rabies free, has recently reported a rabies-like disease in humans caused by exposure to bats.[2] As the rabies virus persists in many animal reservoirs, it may not be possible to eliminate human rabies in the near future. Although human rabies is virtually 100% fatal, it is effectively preventable if state of the art prophylactic measures are instituted, particularly in people exposed to bites from proven rabid animals. Laboratory confirmation of the rabid status of the biting animal becomes important considering the fact that rabies vaccines and immunoglobulins are very expensive and are always in short supply. In countries like India, more than three million people per year are exposed to dog bites and require post-exposure treatment, with a consequential burden on the government finances.[3] However, the majority of these exposures are to animals which later turn out to be normal and healthy. Post-exposure treatment with these expensive biologicals can be avoided once the diagnosis of rabies is ruled out by approved laboratory tests using a brain specimen of the biting animal.

Many tests have been used for the diagnosis of rabies, including detection of Negri bodies, the fluorescent antibody test, the infection of neuroblastoma cells, mouse inoculation and the recently developed enzyme immunoassays and molecular methods.[4] However, with the exception of the demonstration of Negri bodies, all other tests require considerable expertise and expensive equipment and are generally only carried out in reference laboratories. Diagnosis based purely on Negri body demonstration may be erroneous, as both false negatives and false positives are frequently observed.[5]

There is therefore a need to develop a simple and economical test for the diagnosis of rabies which can be routinely used in peripheral centers under field conditions without compromizing on specificity and sensitivity. These tests should also assist in the rapid confirmation of rabies in patients presenting with atypical clinical features. Detection of viral antigens by enzyme immunoassays using reagents blotted on to nitrocellulose or other membranes is not a new technique in diagnostic virology.[6–8] However, use of this procedure in rabies diagnosis has not found wide application. Keeping this in view, this paper describes the development and evaluation of a direct dot blot enzyme immunoassay (DIA) for detection of the rabies antigen in autopsied brain tissues and clinical samples. The results indicate a very high level of correlation with the gold standard technique — fluorescent antibody test (FAT) — and this new test may serve as an economical alternative that can be used routinely under field conditions, not only for diagnosis but also for epidemiological surveys. This test may also be useful for the ante-mortem diagnosis of human rabies.

Materials and methods

Brain samples

A total of 266 brain samples (250 from suspect animals and 16 from humans) were included in this study. The specimens from animals (portions of Ammon's horn and cerebral cortex) were sent to the laboratory preserved in 50% glycerol saline. Of these, 210 samples were from dogs, 30 were from cattle and 10 from cats. Sixteen human brains were also sampled. These were taken fresh after autopsy on patients admitted to the neurological services department of the National Institute of Mental Health and Neuroscience (NIMHANS) and suspected to have died of rabies.

Clinical samples

Cerebrospinal fluid (CSF) and saliva samples of these same patients taken ante-mortem between the

third and the fifth days of illness and preserved at −75 °C were also included. These were tested by dot immunoassay to determine if this test could be used for the ante-mortem diagnosis of human rabies.

Preparation of polyclonal antinucleoprotein antibody and its biotinylation

The rabies nucleoprotein was purified from infected Vero cells (ATCC CCL, 81 obtained from National Centre for Cell Science, Pune, India) using standard procedures described earlier.[9] Briefly, confluent Vero cell monolayers in Roux bottles were infected with Vero cell adapted CVS 11 strain of the rabies virus (obtained from Central Research Institute, Kasauli, India). After 72 hours incubation at 33 °C, the cells were scraped, suspended in sterile phosphate buffer saline at pH 7.2 (PBS) and centrifuged at 20,000 × *g*. The pellet was resuspended in an equal volume of sterile distilled water and was homogenized using a glass Dounze homogenizer. The material was again centrifuged and the clear supernatant was collected. This supernatant was subjected to ultracentrifugation at 150,000 × *g* in a cesium chloride gradient using a Sorvall Discovery 100 S ultracentrifuge for 18 hours. The clearly visible rabies nucleocapsid band was collected by side puncture of the tube and was dialyzed overnight against PBS. The protein content was determined by Lowry's method and purity was confirmed by SDS PAGE, which showed a single band at 57 Kda.

A female New Zealand rabbit (2.5 kgs) was immunized with this protein. Before immunizing the animal, permission was obtained from the institutional animal ethics committee and the animal was housed and maintained as per the regulations in force. The first dose consisted of 400 μg of protein mixed with an equal volume of complete Freund's adjuvant. The subsequent doses were given at fortnightly intervals but mixed with incomplete adjuvant. The antibody titers were checked by indirect immunoflourescence one week after the fourth dose. When the antibody titer was found to be 1:100,000, the rabbit blood was collected by intracardiac puncture after a light ether anesthesia. The serum was separated and the IgG was purified by ammonium sulfate precipitation. The protein content was determined by Lowry's method. The antinucleoprotein globulin was biotinylated using standard procedure[10] using biotinamide N hydroxy succinamide (Sigma, USA. Cat No. B 2643).

Dot blot enzyme immunoassay

The assay was performed using a polyvinylidene difluoride membrane (Biotrace PVDF, Gelman Sciences, USA, Cat No. 8343). The PVDF membrane was cut into the appropriate size and markings of 1 × 1 cm were made with a pencil. The membrane was then put in a plastic tray and treated with 100% methanol for five minutes in the dark. The membrane was stabilized for 30 minutes with a buffer (Tris base 5.8 g, glycine 2.9 g, methanol 200 ml and the volume made up to 1 litre using milli-Q water). Before testing the actual samples, the procedure was standardized using 10% homogenate (in PBS) of rabies-infected mouse brains and normal mouse brains. A distinct dark brown spot developed with rabid mouse brain suspension and there was no color development with normal mouse brain suspension. Subsequently these were used as positive and negative controls for all future tests.

The test brains (portions of Ammon's horn and cerebral cortex) were homogenized (10% in sterile PBS); centrifuged and 3 μl of the clear supernatant was directly applied as a spot on the membrane. The saliva and CSF samples were also applied in a similar manner. For testing these samples, six saliva samples and six CSF samples taken from apparently normal healthy people (not having a previous history of animal bite or rabies vaccination) were also used. The membrane was left at room temperature in a moist chamber for one hour and submerged for 30 minutes in a blocking solution containing 3% skimmed milk powder and 1% normal goat serum in PBS. The membrane was later rinsed three times with PBS and air dried. To each spot of the test material, 3 μl of biotinylated anti-ribonucleoprotein (RNP) antibody was added at a predetermined dilution of 1:500. The membrane was left at room temperature for one hour under moist conditions and washed three times with PBS Tween 20 (PBST). To each spot, 5 μl of streptavidin peroxidase (Sigma, USA, Cat No. S 9420, 1:15,000 dilution) was added and kept at room temperature for 30 minutes. After washing three times with PBST, freshly prepared diamino benzidine (DAB, 0.04% in PBS with hydrogen peroxide 0.1%) was added and the membrane kept in the dark for ten minutes. The reaction was terminated using tap water. A distinct dark-brown colored spot was observed with the positive control and all positive brain samples. There was no color observed with the negative control and negative brain samples (Figure 1).

Fluorescent antibody test (FAT)

This was performed as per the standard procedure advocated by the World Health Organization.[11] The rabies FITC conjugate was obtained from the Central Research Institute, Kasauli (India). For carrying out the FAT, smears were obtained from cut portions

of Ammon's horn and cerebral cortex of brain tissues. The stained slides were observed for fluorescence using a Leitz Diaplan fluorescence microscope.

Mouse inoculation test (MIT)

For detection of the rabies virus in clinical samples like CSF and saliva, the mouse inoculation test[12] was performed using litters of two to three-day-old suckling mice. Permission was obtained from the institutional ethics committee for use and experimentation on animals. The CSF samples were inoculated without any pretreatment with antibiotics but saliva samples were pretreated with gentamicin. The inoculated mice, along with their mothers, were housed and maintained as per regulations in force and observed for 30 days. The rabies infection in the mice developing sickness was confirmed by carrying out a direct FAT on their extracted brains.

Results

Post-mortem brain samples

A total of 266 brain samples were tested by FAT and the same samples were also tested by DIA. The comparative results of these tests are given in Table 1. It can be seen that the concordance between DIA and FAT varied from 96.6 to 100%, depending on the animal species, and the overall concordance was 98.4%. The variation observed probably reflects the stage of illness during which the brain sample was obtained rather than a species difference. The animal brains where DIA was negative but FAT positive had a very scant distribution of viral antigen suggesting that these animals were probably sacrificed earlier in the course of the disease process. By comparison to FAT, the specificity of this DIA was found to be 100%. In this study, 15 animal brains (all dogs) were partially putrefied. In spite of this, the results obtained with these samples also showed 100% concordance, suggesting that DIA can also be used in partially putrefied brain samples.

Ante-mortem clinical samples

Twelve saliva and 12 CSF samples were tested from patients whose diagnosis was confirmed at autopsy by direct FAT on their respective brain tissue samples. Of these, five CSF and four saliva samples were positive by both DIA and MIT, whereas two saliva samples and one CSF sample were positive only by MIT (Table 2). The remaining six samples of saliva and CSF were found to be negative by both the tests. Therefore, the concordance of this procedure with MIT is 83.3% for saliva and 91.6% for CSF. As with the brain samples, no false negative results were observed and therefore the specificity with clinical samples is also 100%. However, the overall sensitivity of this procedure as an ante-mortem diagnostic tool is quite low, about 33.3% in the case of saliva and 43.3% for CSF samples.

Discussion

The detection of viral antigens in various clinical specimens such as stools, respiratory secretions and serum using dot blot immunoassays has proved to be very useful both for diagnosis and in epidemiological studies.[6–8] Use of this type of immunoassay in the diagnosis of rabies has not found wide application.

Table 1 Results of dot immunoassay on animal and human brain samples compared with fluorescent antibody test.

Brain samples	DIA+ FAT+	DIA− FAT−	DIA+ FAT−	DIA− FAT+	Concordance %
Dog brain (*n* = 210)	120/210	87/210	0/210	3/210	98.5%
Cat brain (*n* = 10)	5/10	5/10	0/10	0/10	100%
Cattle (*n* = 30)	16/30	13/30	0/22	1/30	96.6%
Human (*n* = 16)	12/16	4/16	0/16	0/16	100%
Total (*n* = 266)	153/266	109/266	0/250	4/266	98.4%

DIA = dot immunoassay; FAT = fluorescent antibody test.

Table 2 Results of dot blot enzyme immunoassay and mouse inoculation test with cerebrospinal fluid and saliva samples.

Clinical sample	DIA+ MIT+	DIA− MIT−	DIA+ MIT−	DIA− MIT+	Concordance %
Saliva (*n* = 12)	4/12	6/12	0/16	2/12	83.3%
CSF (*n* = 12)	5/12	6/12	0/12	1/12	91.6%

DIA = dot blot enzyme immunoassay; MIT = mouse inoculation test.

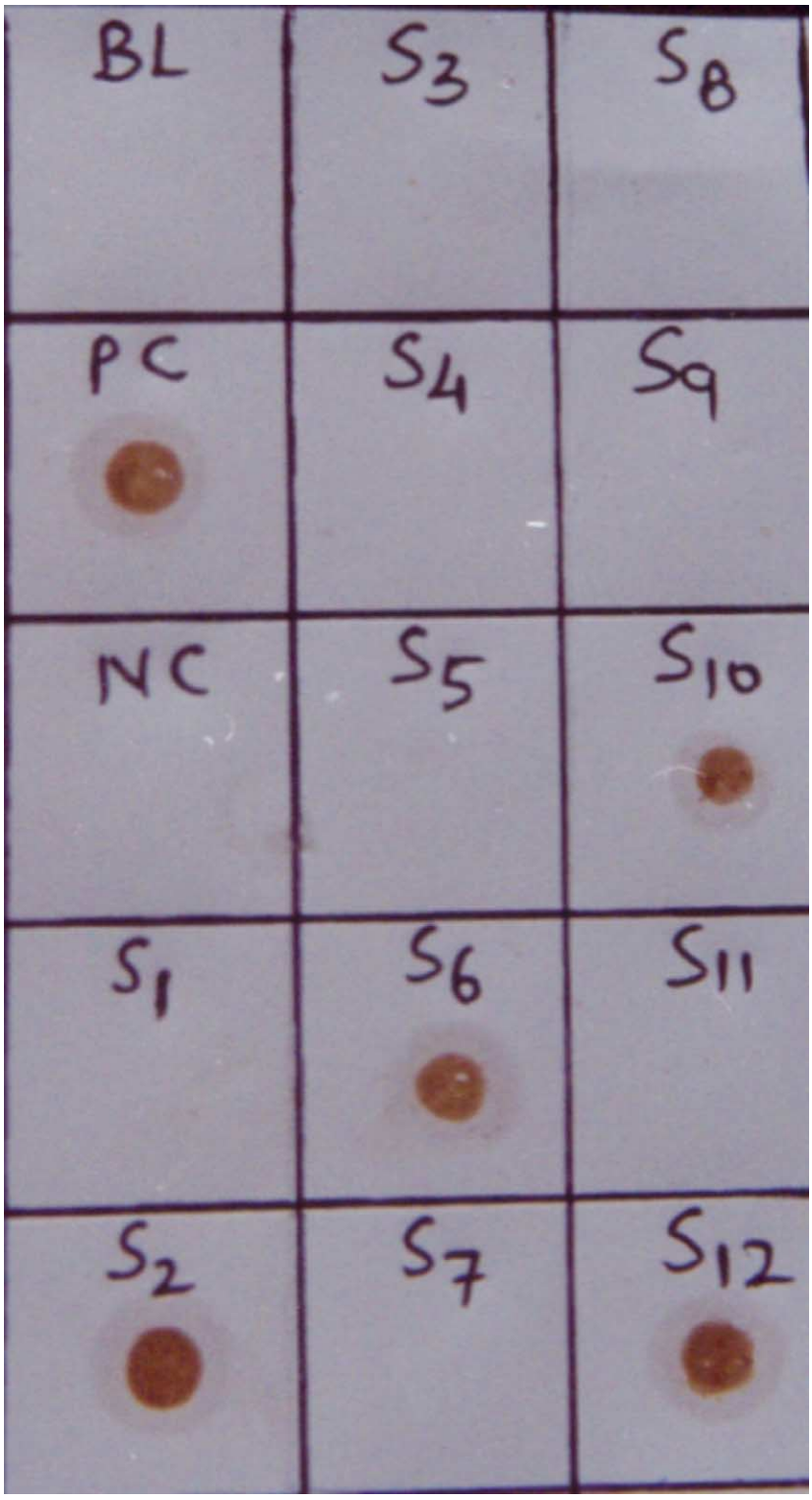

Figure 1 Dot blot immunoassay on a polyvinylidene difluoride membrane for the rapid diagnosis of rabies. BL = Blank; PC = Positive control; NC = Negative control; Positive samples: S_2, S_6, S_{10} and S_{12}; Negative samples: S_1, S_3, S_4, S_5, S_7, S_8, S_9 and S_{11}.

As the World Health Organization is evolving various strategies to control and eliminate human and animal rabies in Asian countries, diagnostic techniques that can be easily applied in the field will provide a true epidemiological picture.[13] Further, most of the animal bites in developing countries like India occur in peripheral districts without laboratory facilities to confirm the rabid status of the animal. Often, the

brains of these animals are sent to central laboratories for diagnosis and post-exposure treatment instituted pending the test report, which may take many days to weeks. Many of these post-exposure treatments could be avoided if the rabid status of the animal could be ruled out by the use of simple tests that could be carried out at small peripheral laboratories. Keeping this in mind, a direct dot blot immunoassay has been developed and evaluated against the gold standard FAT. The assay itself is a simple procedure consisting of the application of the brain homogenate (antigen) as a dot on the PVDF membrane, followed by drying and subsequent detection using biotinylated antinucleoprotein antibody and color development with DAB. The earlier work carried out by Jayakumar et al. used similar principles but they used antiserum to the whole virus and nitrocellulose membrane for blotting[14].

In order to further increase the specificity and sensitivity of the test, the rabies nucleoprotein antigen was purified, the antinucleoprotein antibody was raised and the antibody was biotinylated. As the rabies nucleoprotein is the most abundant viral antigen produced in the infected brain, this rabies-specific antigen can be easily detected using the specific antibody. The results of this study indicate that there is a 97.6% concordance with the gold standard FAT and the specificity of this test is 100%. The test is rapid, does not require costly equipment and the results can be observed by visual examination for the presence (positive) or absence (negative) of distinct dark brown spots. The only specific reagent that is required is the biotinylated antinucleocapsid antibody. This could be prepared in a central reference laboratory and then supplied to peripheral laboratories, which could then routinely perform this test and diagnose rabies. This will not only help in avoiding unnecessary post-exposure treatments but also in performing epidemiological surveys for the prevalence of rabies in animals in specific geographic regions.

Another advantage of this test is that even partially decomposed specimens could be tested, which is not the case with FAT, as interpretation may be difficult in the presence of non-specific fluorescence. This is in accordance with earlier results obtained with other enzyme immunoassays such as rapid rabies enzyme immunodiagnosis (RREID) developed by Perrin et al.[15]

One disadvantage of this test seems to be the occasional false negatives that can be encountered if the animal is killed early in the disease process. In such cases, increasing the concentration of brain homogenate (from 10 to 20% or 30%) may yield positive results. This aspect needs to be further studied.

Another aspect that needs to be evaluated is the ability of this test to detect rabies antigen in clinical specimens like saliva and CSF from patients with suspected rabies encephalitis. Although majority of patients in Asian countries present with typical clinical features of furious rabies, including hydrophobia, atypical presentation with ascending paralysis involving all the limbs and urinary bladder is not uncommon. For instance, in this tertiary care specialized hospital approximately five to seven cases of paralytic rabies are admitted every year. Often the diagnosis is confirmed only after autopsy. Many tests have been advocated for ante-mortem diagnosis including the much-publicized corneal test and frozen section skin biopsy.[16] However, our experience of these tests has not been encouraging. In order to evaluate the detection of viral antigen in clinical specimens like CSF and saliva as a means of ante-mortem diagnosis of human rabies, these samples were tested by DIA with MIT used as a confirmatory test. Although there was good correlation between the results of DIA and MIT, the overall sensitivity of the technique when used with CSF or saliva was lower than when used with brain specimens. This is as expected because the presence of the virus in the CSF of rabies patients is not a constant feature and salivary secretion of the virus is intermittent, requiring testing of several samples. As the specificity of this test was 100%, even with clinical samples, a positive result with this test will help the physician to establish a definitive diagnosis of rabies ante-mortem and help in the appropriate management of not only the patient but also the close contacts.

Conclusion

A simple, rapid, direct dot immunoassay has been developed and evaluated for the detection of rabies antigen in rabies-suspected animal brains. The test has the potential to be used in field conditions, helping not only the institution of timely post-exposure rabies prophylaxis but also facilitating epidemiological surveys for animal rabies in the region. However, the utility of this technique for rapid ante-mortem diagnosis of human rabiesis limited. This test is in the process of being evaluated prospectively with other tests recommended for ante-mortem diagnosis, including the detection of viral nucleic acid in clinical specimens by PCR.

Acknowledgements

The authors are grateful to Dr. S.K. Shankar, Professor and Head, and In-charge, Human Brain Tissue

Repository (HBTR), Department of Neuropathology, NIMHANS, for arranging to conduct autopsy and provide human brain samples.

Conflict of interest: No conflict of interest to declare.

References

1. World Health Organization. *World survey of rabies No. 35*. Geneva: WHO; 2002 p. 4—5.
2. Fraser GC, Hooper PT, Lunt RA, et al. Encephalitis caused by a Lyssavirus in fruit bats of Australia. *Emerg Infect Dis* 1996;**2**:327—31.
3. Ichhpujani RL, Bharadwaj M, Chhabra M, Dutta KK. Rabies in India. In: Proceedings of 4th International Symposium on Rabies Control in Asia. 5—9 March 2001; Merieux Foundation and World Health Organization; 2001. p. 27.
4. Meslin F-X, Kaplan MM. An overview of laboratory techniques in diagnosis and prevention of rabies and in rabies research. In: Meslin F-X, Kaplan MM, Koprowsky H, editors. *Laboratory techniques in rabies*. 4th edition Geneva: WHO; 1996. p. 9—27.
5. Pert DA, Good PF. Pathology of rabies in the central nervous system. In: Bear GM, editor. *The natural history of rabies*. Boca Raton: CRL Press; 1991. p. 163—90.
6. Anand T, Narasa Raju TA, Vishnu C, Venkateswar Rao LV, Sharma G. Development of Dot-ELISA for the detection of human rotavirus antigen and comparison with RNA-PAGE. *Lett Appl Microbiol* 2001;**3**:176—80.
7. Reina J, Padilla E, Alonso F, Ruiz De Gopegui E, Munar M, Mari M. Evaluation of a new dot blot enzyme immunoassay (directigen flu A + B) for simultaneous and differential detection of influenza A and B virus antigens from respiratory secretions. *J Clin Microbiol* 2002;**40**:3515—7.
8. Gentilomi G, Musiani M, Zerbini M, Gallinella G, Venturoli S, Manaresi E. Dot immunoperoxidase assay for detection of parvovirus B19 antigen in serum samples. *J Clin Microbiol* 1997;**35**:1575—8.
9. Dietzschold B. Techniques for purification of rabies virus, its subunits and recombinant products. In: Meslin F-X, Kaplan MM, Koprowsky H, editors. *Laboratory techniques in rabies*. 4th edition. Geneva: WHO; 1996. p. 175—80.
10. Guesdon JG, Ternynck T, Avrameas A. The use of avidin-biotin interaction of immuno-enzymatic techniques. *J Histochem Cytochem* 1979;**27**:1131—9.
11. Dean DJ, Abeleseth MK, Atanasiu P. The fluorescent antibody test. In: Meslin F-X, Kaplan MM, Koprowsky H, editors. *Laboratory techniques in rabies*. 4th edition Geneva: WHO; 1996. p. 88—95.
12. Koprowsky H. The mouse inoculation test. In: Meslin F-X, Koprowsky H, Kaplan MM, editors. *Laboratory techniques in rabies*. 4th edition Geneva: WHO; 1996. p. 80—7.
13. World Health Organization. Strategies for the control and elimination of rabies Asia. Report of the WHO interregional meeting. Geneva, 17—21 July 2001. WHO/CDS/CSR/EPH/2002.8.
14. Jayakumar R, Nachimuthu K, Padmanaban VD. A dot enzyme linked immunosorbent assay (Dot ELISA): comparison with standard fluorescent antibody test (FAT) for diagnosis of rabies in animals. *Comp Immunol Microbiol Infect Dis* 1995;**18**:269—73.
15. Perrin P, Rollin PE, Sureau P. A rapid rabies enzyme immunodiagnosis (RREID): a useful and simple technique for the routine diagnosis of rabies. *J Biol Stand* 1986;**14**:217—22.
16. Hemachuda T, Mitrabhakdi E. Rabies. In: Davis L, Kennedy PG, editors. *Infectious Diseases of the Nervous System*. Oxford: Butterworth Heinemann; 2000. p. 401—44.